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Exhibit 2

TGF-β related genes in development

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Embryonic induction is the process by which signals from one cell population change the developmental fate of another. Polypeptides related to growth factors are one group of molecules mediating many inductive events. Recent data on the embryonic expression and function of signaling proteins related to transforming growth factor β, in both vertebrate and invertebrate systems, have shown that these molecules play important roles in both pattern formation and tissue specification during embryogenesis.

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Introduction

The transforming growth factor (TGF)-β superfamily is one of the largest groups of highly conserved intercellular signaling molecules regulating embryonic growth and differentiation. This superfamily, which currently includes at least 24 members, has several subgroups. The main groups are TGF-βs, *decapentaplegic*-Vg-related (DVR) proteins (including the bone morphogenetic proteins, or BMPs), and activins [1••]. Some members cannot be classified into a specific subgroup, however, and new ones are still being identified.

Over the past year, genetic, biochemical, and functional studies have supported the idea that TGF-β related proteins mediate inductive interactions during development. In some cases, these interactions involve a simple switch in the fate of the responding cells. In other systems, evidence suggests that TGF-β related proteins behave as morphogens, acting over relatively large distances in a graded fashion to differentially specify cell fate. This review includes data from studies in *Drosophila*, *Xenopus*, chick, and mouse, as well as *in vitro* studies with various cell lines.

Structural and biochemical analysis

TGF-β related proteins are synthesized as large pre-pro precursor molecules that are cleaved at an Arg-X-X-Arg site (where X can be any amino acid) to release a carboxy-terminal peptide of 110–140 amino acids, of which 7–9 are conserved cysteine residues. One of these cysteines is involved in intermolecular disulfide bonding to give a biologically active homo- or hetero-dimer [2,3].

Comparison of the crystal structures of three different growth factors, TGF-β2, platelet-derived growth factor (PDGF)-BB, and nerve growth factor (NGF), has recently revealed a common structural motif involving two pairs of β-strands and an arrangement of conserved cysteine residues known as the 'cystine knot' [4]. Thus, although little overall sequence similarity is seen between these growth factors and they form dimers in different ways, they have a similar protomeric organization.

Recently, several new members of the DVR subgroup have been reported; these include nodal, glial-derived neurotrophic factor (GDNF), dorsalin-1, and two proteins which lack the conserved cysteine residue involved in dimerization, namely growth/differentiation factor-3/Vg-related 2 (GDF-3/Vgr-2) and GDF-9 [5,6,7••,8•,9••]. Fig. 1 shows the evolutionary relationships of DVR subgroup members. In some instances, the conservation of sequence means that vertebrate and invertebrate proteins can be functionally substituted; for example, two *Drosophila* proteins, DPP and 60A, induce bone when injected subcutaneously in rat [10]. Conversely, Padgett *et al.* [11] substituted the carboxy-terminal sequence of human BMP-4 with that of DPP to rescue *Drosophila decapentaplegic* (*dpp*) mutant embryos.

The biosynthesis of several TGF-β related proteins has now been studied in transfected cell lines. BMP-2, BMP-4, DPP, 60A, TGF-β1, and DVR-6 are all processed similarly and, as predicted, dimers of cleaved mature region are secreted into the medium ([12–17]; NA Wall, unpublished data). The *in vivo* processing of TGF-β related proteins is much less well understood, however. BMP-1, though co-purified with several BMPs, does not resemble TGF-β, but rather has an astacin metalloproteinase domain and is closely related to the *Drosophila tolloid* gene product [18]. Null mutants of *tolloid* have a phenotype very similar to *dpp* mutants, and *tolloid* and *dpp* expression domains overlap [19]. Studies of *tolloid* mutants suggest that the protein is indirectly involved in proteolytically activating TGF-

Abbreviations

BMP—bone morphogenetic protein; *dpp*—*decapentaplegic*; DVR—*decapentaplegic*-Vg-related; GDF—growth/differentiation factor; GDNF—glial-derived neurotrophic factor; *hh*—*hedgehog*; NGF—nerve growth factor; PDGF—platelet-derived growth factor; TGF—transforming growth factor; *wg*—*wingless*.

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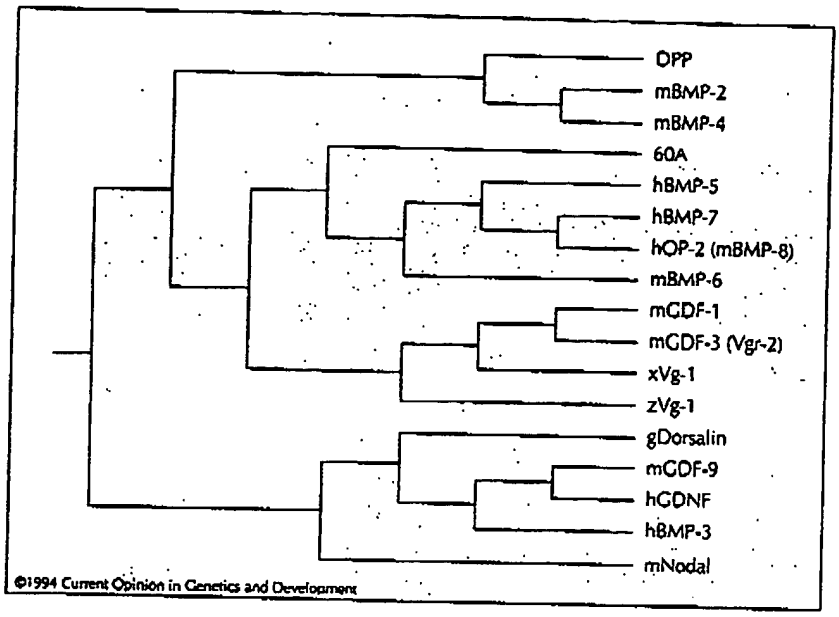

Fig. 1. Phylogenetic relationships of *decapentaplegic*-Vg-related (DVR) proteins on the basis of comparison of carboxy-terminal sequences using the computer program PAUP 3.1 [59]. Similarity is indicated by the horizontal distance of any two sequences from a branch point. m—mouse; h—human; x—*Xenopus*; z—zebrafish; g—*Gallus* (chick); DPP—decapentaplegic; BMP—bone morphogenetic protein; OP—osteogenic protein; GDF—growth/differentiation factor; Vgr—Vg-related; GDNF—glial-derived neurotrophic factor.

β related proteins [20,21]. Recently, a *Xenopus* BMP-1 gene has been cloned and its expression shown to be tightly controlled during early development [22]. This raises the possibility that the activity of TGF-β related factors can be regulated *in vivo* at the level of protein processing.

New TGF-β related ligands continue to be identified, but relatively little is known about their receptors, which fall into two classes, type I and type II, both of which are families of transmembrane serine/threonine kinases. Whereas genes encoding receptors for TGF-β and activin have been cloned, only recently have candidate receptors for DVRs been reported. These include daf-4, a *Caenorhabditis elegans* type II receptor which can bind BMP-2 and BMP-4, and the *Drosophila saxophone* product, which appears to be a type I DPP receptor [23,24].

Genetic analysis of gene function

In the past year, several groups have published exciting new data on the function of *dpp* in *Drosophila*. Among other things, these studies raise the possibility that *dpp* regulates and is regulated by two other secreted growth factors, encoded by *wingless* (*wg*) and *hedgehog* (*hh*). As both *hh* and *wg*-type (Wnt) genes exist in vertebrates, work on *dpp* in *Drosophila* provides an important paradigm for the understanding of TGF-β related gene function in higher organisms.

The *dpp* gene is necessary for several different morphogenetic processes during *Drosophila* embryogenesis, including dorsal/ventral patterning of the embryo, establishment of the proximo-distal axis of appendages, eye development, and midgut morphogenesis [25–27]. In dorsal/ventral patterning, evidence suggests that the DPP protein acts as a morphogen regulating dorsal cell fate [28]. Wharton *et al.* [29••] have demonstrated that increasing the number of *dpp* genes in either mutant or wild-type embryos results in the formation of an increasing number of dorsal type cells in a dose-dependent manner.

During midgut development, DPP is made in the visceral mesoderm and induces changes in gene expression in the adjacent endoderm, thus providing a model for understanding epithelial/mesenchymal interactions in higher organisms. Studies have shown that *dpp* transcription in the mesoderm is activated by the HOM gene *Ultrabithorax* (*Ubx*) and repressed by *Abdominal-A* (*Abd-A*) [30•] (Fig. 2a). In both cases, regulation is direct and involves multiple DNA-binding sites in a 5′ upstream region of *dpp* [31•,32]. In the endoderm, DPP switches on the HOM gene *labial*, which has a DPP-response element in its 5′ upstream region [33]. These studies clearly show that *dpp* acts during development both upstream and downstream of homeotic genes.

Studies on eye morphogenesis demonstrate yet another key role for *dpp* during *Drosophila* development [34••]. In this case, *dpp* is expressed in the morphogenetic furrow, a wave of organizing activity that moves across the eye imaginal disc (Fig. 2b). Transcription of *dpp* is regulated by the extracellular signaling molecule encoded by *hh*, and DPP induces morphogenesis in front of the furrow [35••]. In the leg disc, *dpp* is also regulated by *hh* [36••] and cooperates with *wg* in the regulation of the homeobox gene *aristaless*, which then acts as an organizer for proximal/distal patterning [37••] (Fig. 2c). In this case, at least three signaling molecules, hh, DPP, and Wg, coordinate to generate patterning in an epithelial sheet.

Drosophila is not the only system providing genetic clues to the biological importance of TGF-β related genes. Important contributions are now beginning to come from mice with mutations or targeted disruptions of genes in this family. The *short ear* (*se*) mutant has deletions or rearrangements in the gene for BMP-5 that are associated with loss of specific skeletal structures, indicating

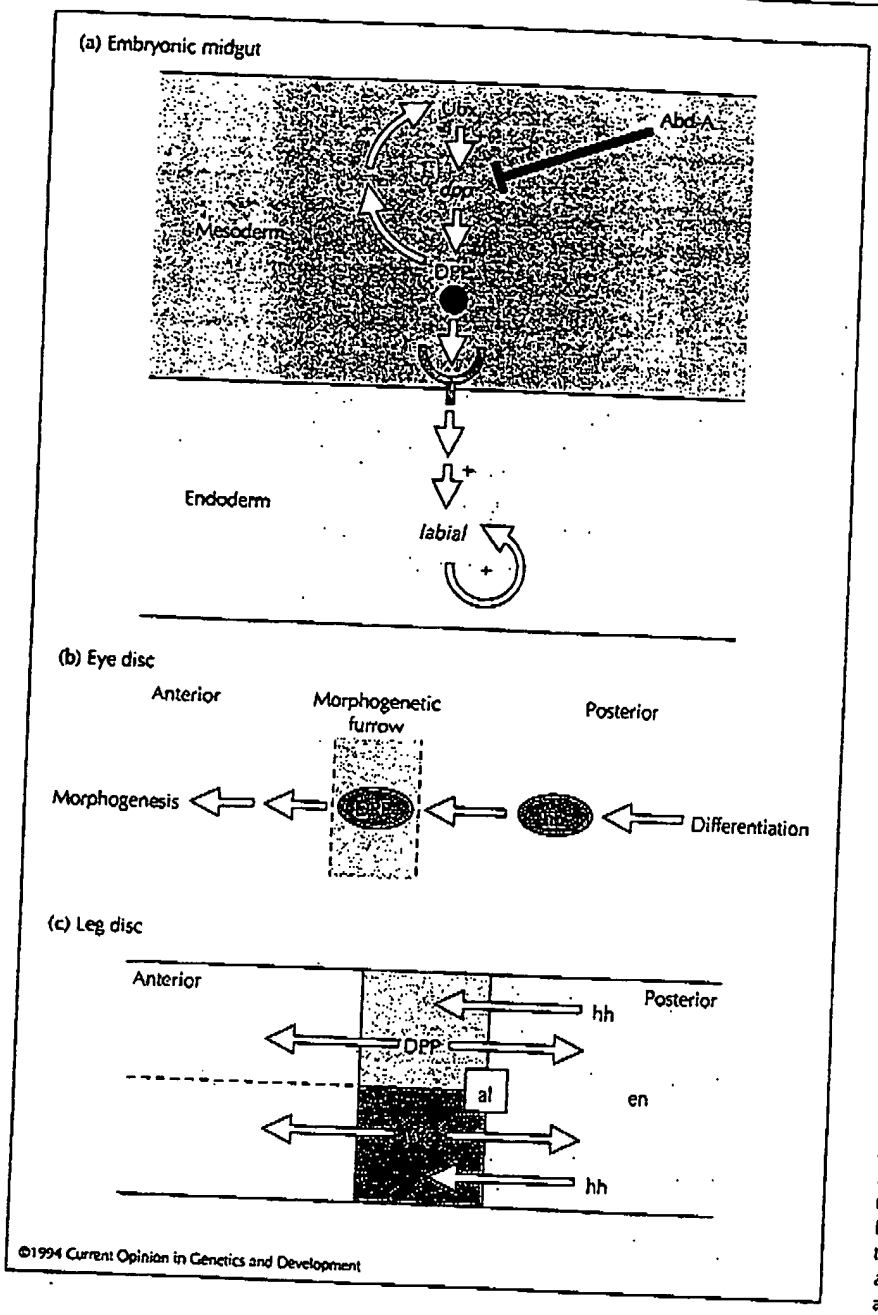

Fig. 2. Schematic models for some of the interactions of *decapentaplegic* (*dpp*) with transcription factors and other signaling molecules during *Drosophila* development. (a) During visceral mesoderm/endoderm interactions in embryonic midgut development, Ubx directly and positively (+) regulates *dpp* transcription, whereas Abdominal-A (Abd-A) inhibits it (–). The DPP protein elicits an intracellular signaling pathway in the endoderm that positively regulates *labial* transcription. DPP also indirectly maintains *Ubx* expression. Other interactions and regulatory factors are likely [30•,31•,33]. (b) During eye disc development, differentiated cells posterior to the morphogenetic furrow express *hedgehog* (*hh*). The diffusible hh signal regulates *dpp* expression in the cells of the morphogenetic furrow (anterior). DPP protein then mediates, directly or indirectly, morphogenesis anterior to the moving furrow [34••,35••]. (c) During leg disc development, cells expressing *engrailed* (*en*) in the posterior leg disc express *hh*, and hh diffuses anteriorly to adjacent cells to regulate *dpp* and *wingless* (*wg*) expression in dorsal and ventral regions, respectively. The interaction of DPP and Wg results in the focal transcriptional activation of the homeobox gene *aristaless* (*al*) which is thought to define a distal organizer [36••,37••].

a role for the gene product in skeletogenesis [38]. In the case of the mouse mutation 413-d, studies have shown that it results from a retroviral insertion in the TGF-β related gene *nodal*. During gastrulation, homozygous mutants are unable to form axial structures, including organized dorsal mesoderm and notochord [7••,39,40]. *In situ* hybridization studies have revealed that *nodal* RNA is expressed in a small group of cells around the node, but the precise role of the protein in axial patterning is not yet known.

Targeted disruptions of TGF-β1, inhibin α, and activin/inhibin βB genes all result in aberrant phenotypes in homozygous mutant mice. Mice that are homozygous null mutant for TGF-β1 are viable for a few weeks before dying from a 'wasting' syndrome [41]. These results indicate that the embryo does not need to produce TGF-β1 for prenatal development, although the mother may provide some TGF-β1 protein in the uterus and milk. Mice deficient for inhibin α are also viable, but develop gonadal stromal tumors, suggesting that one function of the protein is to regulate or suppress stromal cell proliferation in this organ [42]. Targeted disruption of the related gene encoding activin/inhibin βB has also been reported [43]. Homozygous null mutants are viable, but females have reduced fertility, indicating a role for activin/inhibin βB in reproductive function. However, the fact that some viable offspring are born to these females

eliminates activin βB from playing a role in embryonic pattern formation.

Functional analysis

Besides genetic studies, various groups have used purified peptides and/or RNA misexpression to explore the function of TGF-β related proteins in a variety of *in vitro* assays. Overexpression of DVR-4/BMP-4 in *Xenopus* embryos causes induction of posterior and ventral types of mesoderm, whereas activin can induce dorsal types of mesoderm. In both cases, the proteins appear to act in part by regulating the expression of anterior–posterior-specific homeobox genes [44–48]. On the basis of the localization of its mRNA to the vegetal pole, Vg-1 was a prime candidate for an *in vivo* mesoderm inducer [49]. Initial experiments involving misexpression of Vg-1 mRNA did not show any effect on mesoderm formation, however. Although Vg-1 protein was produced, it was not processed to release the active, carboxy-terminal homodimer [50]. Recently, two groups have used constructs encoding chimeric proteins (BMP-2/Vg-1 and BMP-4/Vg-1) that circumvent the block on protein processing and have shown that mature Vg-1 does induce dorsal mesoderm [51••,52••]. Moreover, chimeric Vg-1 protein can rescue embryos ventralized by UV irradiation, suggesting that it acts by induction of the Nieuwkoop center. It now appears, therefore, that Vg-1 is an endogenous dorsal mesoderm inducer in *Xenopus* embryos, but is tightly regulated at a post-transcriptional level.

Two *in vitro* systems exploring epithelial/mesenchymal interactions in vertebrates have implicated a mediating role for BMP-4 and BMP-2. Vainio *et al.* [53••] have demonstrated that during tooth development, BMP-4 protein, which is initially expressed in presumptive dental epithelium, induces expression of several genes, including the endogenous BMP-4 gene, in adjacent mesenchyme. In the limb bud, where the apical ectodermal ridge influences the proliferation and differentiation of the underlying mesenchyme, Niswander and Martin [54••] have reported that BMP-2, which is transcribed in the apical ectodermal ridge, attenuates the proliferation of mesenchymal cells by fibroblast growth factor-4, a growth factor produced in the posterior apical ectodermal ridge.

In addition, BMP-2 modulates the differentiation of C3H10T1/2 cells, a mesenchymal stem cell line that can differentiate into myoblasts, chondrocytes, osteoblasts, and adipocytes. When treated with recombinant BMP-2, C3H10T1/2 cells differentiate into chondrocytes and osteoblasts at high protein concentrations and into adipocytes at low concentrations [55]. No myoblasts form in response to addition of BMP-2. In fact, Murray *et al.* [56] have reported that BMP inhibits expression of four myogenic determination genes, encoding myogenin, MyoD, herculin, and myf-5, in two myogenic cell lines. These results suggest that, *in vivo*, one factor regulating the differentiation of multipotent mesenchymal stem cells into different mesodermal phenotypes is the local concentration of DVR protein to which they are exposed, either during embryogenesis or during processes such as wound healing and tissue repair.

Evidence for the patterning activity of TGF-β related proteins is also found in the developing nervous system. The *dorsalin-1* gene is normally transcribed in the dorsal roof plate of the chick embryonic spinal cord. When dorsalin-1 protein is added to cultured spinal cord explants, differentiation of neural crest cells is enhanced, whereas differentiation of ventral motor neurons is inhibited. It appears, therefore, that dorsalin-1 regulates the fate of dorsal cell types in the embryonic neural tube. A distantly related protein, GDNF, is capable of acting as a neurotrophic factor by specifically promoting the survival of midbrain dopaminergic neurons *in vitro*. Although several other TGF-β members are expressed in the nervous system at various times during embryogenesis, it is not yet known precisely how they function [15,57,58].

Conclusions

TGF-β related genes encode a large and growing family of structurally related secreted signaling molecules. Expression studies reveal that many of these genes are active in embryonic tissues undergoing inductive interactions and patterning. Genetic and functional studies support the idea that TGF-β related proteins mediate these events by acting upstream and downstream of transcription factors and other growth factors.

Future research will continue to examine the functional roles of these proteins during development. Targeted gene disruption in mice and interbreeding of different mutant lines will help to reveal which developmental processes require members of this superfamily family, and the production of purified proteins and antibodies will provide tools for elucidating the mechanisms involved. Also, identification of more receptors will facilitate our understanding of the signaling pathways activated by TGF-β related proteins. In fact, investigating these intracellular pathways appears to be one of the most important future directions to be taken in studying the role of TGF-β related genes during development. Given the high degree of sequence identity between members of the TGF-β superfamily, it will be interesting to determine the specificity or promiscuity of ligand–receptor interactions and to understand how the same ligand may elicit very different responses in different embryonic tissues. Finally, more data are needed about the relative roles of gene transcription, protein processing and degradation, and extracellular binding of mature protein in regulating the availability of ligand to receptors in responding tissues.

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